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Cytokinin and the regulation of a tobacco metallothionein-like gene during copper stress

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Higher plant metallothioneins are suspected of reducing metal-induced oxidative stress and binding copper and zinc cofactor metals for delivery to important apometalloproteins. A metallothionein-like gene (MT-L2) cDNA clone pCkn16A1 (Accession U35225) was cloned from heat-shock-induced *Nicotiana plumbaginifolia* containing the heat-shock-inducible isopentenyl transferase (*ipt*) gene (HS-*ipt*). *Ipt* expression in plants leads to enhanced cytokinin biosynthesis. In mature leaves of non-transformed *N. plumbaginifolia* and *Nicotiana tabacum*, copper stress caused a significant loss of MT-L2 mRNA transcripts. Under non-stressed conditions, HS-*ipt* induced (*N. plumbaginifolia*) or light-induced (SSU-*ipt*) (*N. tabacum*) plants had higher MT-L2 transcript levels than non-transformed or transformed (GUS) controls. Unlike control plants, copper stress did not reduce MT-L2 mRNA levels in the cytokinin accumulating transgenic plants. Enhanced cytokinin production also led to lower lipid peroxidation compared with controls under non-stressed and copper-stressed conditions. Greater MT-L2 transcript levels and protection against oxidative events prior to or during copper stress could contribute to the observed eight-fold accumulation of copper in mature leaves of *ipt* expressing plants compared to non-transformed plants. Expression of this tobacco MT-L2 mRNA may be modulated directly by cytokinin or indirectly as a consequence of cytokinin-mediated antioxidant activity.

Introduction

Metallothioneins (MTs) are small molecular weight proteins that contain cysteine-rich N *and* C termini. MTs are classified Class I or Class II based on the arrangement of cysteines within the peptide (Klassen et al. 1999). Most fungal and plant-encoded MT-like (MT-L) genes are similar to those of Class II, containing N-terminal Cys-Cys and Cys-Xaa-Xaa-Cys residues. Differing arrangements of cysteines amongst plant MT and MT-L genes may reflect differences in protein function (Cobbett and Goldsbrough 2002). In wheat, the Ec protein is the only verified plant-encoded MT to be extensively described (Kawashima et al. 1992). However, within the plant kingdom there are numerous and diverse MT-L genes encoding cysteine-rich proteins that resemble either mammalian MTs or the wheat Ec protein.

Animal and yeast MTs are transcriptionally activated, encode MT proteins that bind Zn and Cu, and play important roles in nutrition and metal toxicity/tolerance (Hamer et al. 1985, Thiele 1992, Jensen et al. 1996, Winge et al. 1998). Developmentally regulated, MTs accumulate in a tissue-specific manner and are positively correlated with copper tolerance (Hamer et al. 1985, Freeman and Peisach 1989). MTs have also been implicated in the regulation of ATP production (Ye et al. 2001).

The function of one class of MTs, the MT-L2 genes in plants is at best unclear. MT-L2 mRNAs generally accumulate at specific developmental stages and increase in response to copper and osmotic stress, viral attack, senescence, and wounding (Choi et al. 1996, Rauser 1999, Van Hoof et al. 2001, Guo et al. 2003). Surprisingly, some plant species demonstrate MT-L2 mRNA

Abbreviations – ATA, aurintricarboxylic acid; CHX, cycloheximide; HS promoter, heat-shock promoter from *Drosophila melanogaster*; *ipt*, isopentenyl transferase originally from *Agrobacterium tumefaciens*; SSU promoter, promoter of the pea small subunit of ribulose 1,5-bisphosphatecarboxylase/oxygenase from *Pisum sativum*.

levels that are unchanged or declined following copper stress (Zhou and Goldsbrough 1994, García-Hernández et al. 1998, Rauser 1999). Considering these reports, and because of a correlation between MT-L2 proteins and their mRNA levels (Murphy et al. 1997), it is uncertain how metal stress can enhance, depress or leave unchanged MT-L2 transcript levels depending on the particular plant species.

Plant development may be a process that co-ordinates MT-L2 production in higher plants. A number of reports have recently focused on several MT-L2 genes and their potential role(s) during development (Page et al. 2001, Guo et al. 2003). A developmentally co-ordinated suite of *Mesembryanthemum crystallinum* stress-responsive genes was found induced by cytokinin (Thomas and Bohnert 1993, Thomas et al. 2004). Exogenously added cytokinin also increased the expression of several MT-L2 species in leaves of the Ice Plant (unpublished). Cytokinins, depending on the dose, are able to impede or enhance senescence (Smart et al. 1991, Smigocki 1991, Thomas et al. 1995a, b). As plants senesce, copper, zinc and other essential micronutrients are re-mobilized to the developing seeds, leading to programmed cell death in vegetative tissues (Himelblau and Amasino 2001). MT-L2 mRNAs accumulate during senescence (Page et al. 2001), their MT-L2 proteins perhaps assisting developing embryos by protecting them from copper-initiated free radicals and lipid peroxidation (Sandermann and Böger 1980, Elstner et al. 1988). Important in seed development, endogenous cytokinin was detected in high levels in high-viability seeds whereas the cytokinin was oxidized to adenine in low-viability seeds (Gidrol et al. 1994). One relationship of cytokinins to MT-L2 levels (protein and mRNA) may be cytokinin-enhanced levels of MT-L2 transcripts and proteins which protect the embryos against coincidental metal-activated reactive oxygen species (ROS) and cellular damage (Wong et al. 2004).

The objective of this work was to characterize the expression patterns of a tobacco metallothionein-like gene (MT-L2) (Accession U35225) in response to metal stress in control and cytokinin over-expressing tobacco plants. The MT-L2 gene was recovered from a cytokinin induced *Nicotiana plumbaginifolia* cDNA library (Harding and Smigocki 1994). Unlike many plant species that accumulate MT-L2 mRNAs in response to copper stress, we found tobacco down-regulated this MT-L2 gene after a copper challenge. However, when cytokinin over-expressing tobacco plants were stressed with $CuSO_4$, the MT-L2 mRNA levels were maintained in pre-stressed conditions and copper metal was accumulated in the older leaves (compared to controls). We propose that in response to copper stress, MT-L2 mRNA levels may increase, decrease or remain constant in a particular plant species depending on the endogenous plant cytokinin status. Regulation of MT-L2 mRNA may be exerted by cytokinin directly via transcription or signal transduction (phosphorylation) or, as a general antioxidant effect. Patterns of expression of this second distinct tobacco MT-like gene can also be compared with the other described tobacco MT-L2 gene (from *Nicotiana glauca*) (Choi et al. 1996). Furthermore, this information is relevant because tobacco is frequently the transgenic host for mammalian and yeast MT over-expression studies (Evans et al. 1992, Elmayan and Tepfer 1994, Thomas et al. 2003).

Materials and methods

Plant material and cDNA isolation

Cytokinin up-regulated cDNAs were recovered from a cDNA library using a heat-shock (HS)-induced subtractive probe approach (Harding and Smigocki 1994). Plants were grown on agar medium and heat shocked at 45°C for 1 h in light (200 μEinstein $m^{-2} s^{-1}$), and then returned to 26°C. One HS-*ipt* responsive clone, pCkn16A1 (Accession U35225) was identified as a putative MT-L2 gene.

Nicotiana plumbaginifolia plants grown on agar medium (Harding and Smigocki 1994) were used for metal stress experiments. Leaf strips were placed in Murashige and Skoog (MS) liquid medium and the culture shaken at 120 r.p.m. for 30 h. Metal concentrations in stress experiments were 50–1000 μ*M* in a liquid culture system (Zhou and Goldsbrough 1994).

Seeds from self-fertilized (selfed) T_2 generation lines of transgenic HS-*ipt N. plumbaginifolia* (Smigocki 1991) and SSU-*ipt Nicotiana tabacum* (cv. SR-1) (Thomas et al. 1995a, 1995b) (the latter known as B1) were surface sterilized and germinated on 100 μg ml^{-1} kanamycin, MS medium, 1% sucrose and 8 g l^{-1} Bactoagar (Difco Laboratory, Detroit, MI). Cytokinin over-expression was induced by heat (Harding and Smigocki 1994) or light (Thomas et al. 1995a, b). For a transformed control, a tobacco line homozygous for the gene encoding β-glucuronidase (*uidA*) (or GUS gene) fused downstream of the 35S-promoter from cauliflower mosaic virus was used. *Nicotiana tabacum* plants were grown in 500 cm^3 potting mix (Scotts Co., Marysville, OH) for 4–6 weeks in a growth chamber (24–27°C, 16 h light at 150–200 μEinstein $m^{-2} s^{-1}$).

RNA accumulation

RNA was isolated from *N. plumbaginifolia* as described (Harding and Smigocki 1994). For *N. tabacum*, RNA was extracted from fresh or frozen tissue in 2 m*M* aurintricarboxylic acid (ATA), 150 m*M* NaCl, 100 m*M* Tris pH 8, 10 m*M* EDTA with 1% SDS. After the addition of 0.5-mm glass beads (BioSpec Products Inc., Bartlesville, OK), the solution was vigorously vortexed, an equal volume of Tris-buffered phenol added and the mixture periodically vortexed at room temperature several times within 15 min. Following centrifugation, the supernatant was extracted with 1/2 volume chloroform : isoamyl alcohol (95 : 5 v/v), re-centrifuged, and the aqueous RNA fraction brought to 2 *M* $LiCl_2$. Following a second $LiCl_2$ precipitation, the RNA was dissolved in water, fractionated on a 1.2% formaldehyde agarose gel

and the gel blotted on Genescreen Plus (NEN, Greenfield MA) or Zeta Probe (Bio-Rad, Hercules, CA).

For expression studies in *N. tabacum*, polymerase chain reaction (PCR) M13 forward and M13 reverse sequencing primers (Gibco/BRL, Bethesda, MD) were used to recover tobacco MT-L2 cDNA from the cDNA clone (pCkn16A1). PCR conditions were 95°C for 1 min, 55°C for 1 min, and 68°C for 1 min, repeated for 30 cycles. For expression studies in *N. plumbaginifolia*, MT-L2 specific upstream and downstream primers were used to amplify a 2106-bp fragment (upstream primer 5′-CATGTCTTGCTGCGGAGGAA-3′ and downstream 5′-CAGGTGTCAGTTTGATCCACA-3′). For DNA probes to the RNA blots, the random primer method with α-dATP32 was used, with hybridization in 50% formamide at 42°C. Washes were at 65°C in 2×, 1× and 0.1× SSC (150 mM sodium chloride and 15 mM sodium citrate pH 7.0) buffer with 0.1% SDS. Blots were exposed to Kodak XOMAT X-ray film (Eastman Kodak Co., Rochester, NY).

The 18S rDNA from control genomic *N. tabacum* DNA was recovered using PCR primers previously described (Thomas et al. 2003). Generally the 18S rDNA probe was also hybridized to the RNA blots following MT-L2 hybridization and X-ray film development to provide a comparison of RNA loading per lane and to compare the relative MT-L2 hybridization. In addition, stained RNA gels were used to determine not only the prevalence of the 18S RNA, but also the integrity of the RNA preparation as a whole.

In some comparative RNA expression experiments, the 18S ribosomal gene from *Xenopus laevis* was used (Harding and Smigocki 1994). Quantitative measurements of beta particle emissions from hybridizations to the MT-L2 and 18S rRNA genes were done using a betascope image processor (ATC Diagnostics, Framingham, MA). Counts per minute (cpm) of MT-L2 were relative (normalized) to the 18S rRNA cpm in the same sample lane.

DNA analysis

DNA isolation from liquid N_2 ground plant tissue followed described methods (Thomas et al. 2003) with a 30-min RNase treatment (1 μg ml^{-1}) at 25°C. After restriction digestion, 5 μg DNA was separated on 1.0% (w/v) agarose and blotted to Qiabrane (Qiagen, Valencia, CA). The MT-L2 cDNA was isolated and labelled with α-dATP32 (random primer method), denatured and hybridized to the DNA blot at 42°C in buffer with 50% formamide. Blots were washed at 55°C and exposed as mentioned above. It is of note that this hybridization stringency is lower that that used for RNA blots (see above).

Estimates of mRNA stability

A translation-block method using cycloheximide (CHX) (DeRocher et al. 1998) was used to estimate mRNA stability. Six-week-old-leaves (ninth or tenth leaf from the plant base) were excised, briefly surface sterilized in 10% (v/v) bleach followed by several washes in sterile water, and 1–1.5 cm squares dissected and floated on 1/10 MS medium. CHX was added at 1 μg ml^{-1} and leaves incubated for 2 h at 25°C. Following washes in 1/10 MS medium, CHX was added back to some samples while others did not receive additional CHX for 2 additional hours. The tobacco 18S rDNA used for control hybridization was previously described (Thomas et al. 2003).

Copper stress and plant analysis

GUS or SSU-*ipt* kanamycin resistant *N. tabacum* seedlings were transferred to soil and grown for approximately 5 weeks. Soil-grown plants were then stressed for a 1-week period. A 4-m*M* copper sulphate solution was prepared in water and the pH maintained at approximately 5.5 with 0.1 *M* KOH or HCl. On alternating days during the stress week, 100 ml of either copper or water solution was added to plants in 500 cm^3 potting soil. All samples were taken for analysis during the second half of the light period after 7 days. Importantly, 4 m*M* $CuSO_4$ would be in great excess for *in vitro* or hydroponic cultures. In experiments with soil-grown plants, copper solution run-off and copper absorption to the soil required a 4-m*M* dose of $CuSO_4$ to demonstrate increased proline biosynthesis, chlorosis, and other markers of metal stress in mature non-flowering tobacco plants within 7 days (Thomas et al. 1998) and data not shown.

Copper determination

For each treatment (unstressed or copper stressed), duplicate plants were grown, stressed and analysed for copper. Generally whole leaf or above-ground seedlings (shoots and leaves) were used for copper analysis. Plant samples were dried, ashed at 550°C in a muffle furnace for 3 h, hydrolysed in 3 *M* HCl for 2 h and total copper determined using a PerkinElmer Boston, MA Model 3100 atomic absorption spectrophotometer (Thomas et al. 1998). Data represented the mean copper values from at least six plants (three separate experiments with duplicate plants per treatment) and standard error. Copper standards were obtained from Custom Grade Standards (Inorganic Ventures, Inc., Lakewood, CA). Copper data was analysed statistically using the INSTAT statistical program (GraphPad, San Diego, Software).

Lipid peroxidation

Lipid peroxidation analysis was based on malondialdehyde (MDA) levels (DePaula et al. 1996). Leaf samples (0.25 g FW) were homogenized in a mortar and pestle in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA), then centrifuged at 10 000 × *g* for 10 min. From the supernatant, 1 ml was added to a tube and mixed with 4 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v)

TCA. Samples were incubated at 90°C for 30 m, cooled, and the optical density was measured at 532 and 600 nm. The concentration (m*M*) of MDA g^{-1} tissue was estimated from the 155 m*M* MDA extinction coefficient (DePaula et al. 1996). Data represents the mean MDA values from the same plants used in copper determinations.

Results

Cloning of the MT-L2

The screening procedure of Harding and Smigocki (Harding and Smigocki 1994) was designed to select shoot-specific cytokinin-induced transcripts within 4 h of cytokinin production. One clone, pCkn16A1, contained an open reading frame that predicted a metallothionein-like (MT-L2) protein. The nucleotide sequence contained 462 base pairs with the ORF spanning nucleotide 72–292 (Accession U35225) and a predicted molecular mass of 7100 Da.

Unlike the non-transformed *N. plumbaginifolia* plants, the HS-*ipt* transformed line had detectable MT-L2 mRNA prior to heat shock (data not shown). Post-heat shock, the accumulation of the MT-L2 transcript increased in both normal (non-transformed) and HS-*ipt* plants, with expression being higher in the transgenic plant (data not shown). No differences in copper content were observed between non-stressed and heat-shocked stress treatments in either non-transformed or HS-*ipt* plants up to 48 h post-heat shock (data not shown).

The pCkn16A1 (Accession U35225) derived amino acid sequence closely resembled a metallothionein-like gene. Sequence alignment using CLUSTALW AND SEQPUP (Gilbert 1990–96) indicated that several previously described MT-L2 genes of tobacco were all very similar. In a comparison of the DNA sequences within the open reading frame, *N. plumbaginifolia* (Accession U35225) is closely related to *N. tabacum* (Accession AJ299253), differing in 13.8% of the nucleotides within the open reading frame. Accession U46543 of *N. glauca* and *N. plumbaginifolia* (U35225) differed by 53.3% of the nucleotides within the same encoded region. More importantly and in regard to the amino acid sequence, the MT-L2 gene of *N. plumbaginifolia* was 85.1 and 55.1% identical to *N. tabacum and N. glauca*, respectively. Most of the identity between the *N tabacum* and *N. plumbaginifolia* MT-L proteins was found within the first 30 amino acid residues.

The pCkn16A1 MT-L2 gene was used as a probe on Southern blots prepared with either *N. tabacum* and the *N. plumbaginifolia* genomic DNA. Examination of the http://www.ncbi.nih.gov BLAST and TIGR http://www.tigr.org web sites did not reveal sequences for genomic tobacco MT clones. Therefore, exact gene family member determinations were not possible using the MT-L2 cDNA because we were unable to predict restriction enzyme sites within MT-L2 introns. Introns are present in at least one MT-L2 (Wong et al. 2004). Acknowledging the difficulty of estimating MT-L2 gene family members, when genomic Southern blots were probed with the tobacco MT-L2 cDNA, the cross-reactivity of the cDNA probe at a low hybridization stringency (washed at 55°C) resulted in several hybridizing bands in both *N. tabacum* and *N. plumbaginafolia*. When the cDNA probe hybridization stringency was increased as in the Materials and Methods (washed at 65°C), improved specificity of hybridization (one or a small number of bands) was observed.

Metal effects on MT-L2 expression

Expression of MT-L genes in higher plants is often measured in leaves. Because tobacco leaves develop at different chronological times, examination of MT-L2 expression per leaf number was initiated. Analysis of MT-L2 gene expression in the GUS transformant indicated that MT-L2 mRNA was produced in most leaves at similar levels (Fig. 1A). When plants were treated for 7 days with 4 m*M* $CuSO_4$, MT-L2 mRNA levels declined dramatically (Fig. 1B). Using MT-L2 probes of similar specific activity and equal amounts of RNA, autoradiograph exposure of the MT-L2 unstressed blot (Fig. 1A) was 2 days whereas the MT-L2 stressed blot (Fig. 1B) was exposed for 21 days.

Many higher plants have multiple MT-L2 genes (Cobbett and Goldsbrough 2002, Wong et al. 2004). Therefore, we cannot exclude cross hybridization of related MT-L2 genes in these RNA experiments. However, because of the MT-L2 probe specificity in Southern blots when hybridization stringency was enhanced (as described in the Materials and Methods) the observed overall trends in MT-L2 mRNA levels can be qualitatively inferred. For example, copper stress resulted in a loss of a previously observed MT signal (Fig. 1A and B). This result argues that one or a small subset of MT-L2 transcripts previously hybridizing to the MT-L2 cDNA probe did vanish from the mRNA pool following copper stress. Other methods such as semiquantitative RT-PCR or microarray measurements could help support this observation.

MT-L2 expression was also analysed in *N. plumbaginifolia* leaf segments floating on culture medium to more accurately assess the metal dose to the response without the metal interacting with the soils. After treatment with 100 μ*M* $AlCl_2$, 50 μ*M* $CuSO_4$, 100 μ*M* $CdCl_2$, or 1000 μ*M* $ZnCl_2$ for 30 h, MT-L2 mRNA expression declined in comparison with the untreated control (Fig. 2). Cd and Zn were particularly effective at reducing the levels of the MT-L2 transcripts. In mature non-flowering *N. plumbaginifolia* plants, the MT-L2 mRNA transcript accumulated in all the vegetative parts with slightly greater accumulation in stems and the outer stem tissue (data not shown).

Cycloheximide is thought to restrict the rate of protein elongation, allowing longer ribosome–mRNA association and subsequent protection of mRNA from cytoplasmic localized RNase (DeRocher et al. 1998). In order to determine the stability of the MT-L2 mRNA, leaf

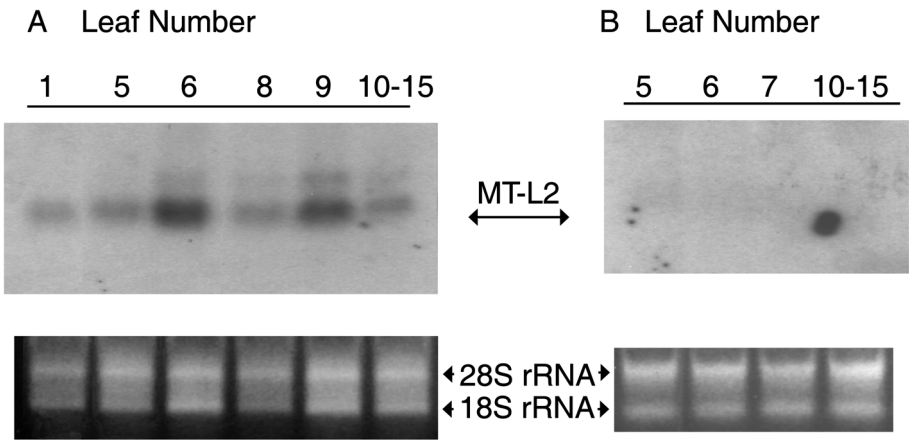

Fig. 1. Expression of MT-L2 mRNA and effects of copper in GUS transformant *N. tabacum* cv. SR1 leaves. A, RNA from a T-3 generation GUS unstressed plant probed with the tobacco MT-L2 gene. B, Hybridization of the tobacco MT-L2 gene to RNA from the same T-3 GUS line as A after 7 days of copper stress. Total RNA loading is depicted as an ethidium bromide-stained gel photograph below A and B, respectively. Higher leaf numbers indicate younger leaves. Leaves 10–15 were very small and pooled. This experiment was repeated and similar results were obtained (data not shown).

sections of *N. tabacum* were treated with cycloheximide (CHX) for 4 h or a 2-h cycloheximide application followed by a wash and 2-h incubation in the absence of cycloheximide. Increased prevalence of MT-L2 mRNA levels was observed with cycloheximide incubation of 4 h or 2 h + 2 h chase compared with untreated controls (Fig. 3). These results indicated the MT-L mRNA accumulated in the presence of CHX. Using a similar approach, plant expressed GUS mRNA and a yeast metallothionein mRNA demonstrated stability of between 1 and 3 h (DeRocher et al. 1998, Thomas et al. 2003).

Cytokinin production increases MT-L2 transcript levels and long-term copper accumulation

The growth habit of the HS-*ipt* and SSU-*ipt* transgenic plants was similar to GUS transformants and non-transformed controls. Both types of transgenic *ipt* plants were somewhat shorter and demonstrated more branching than controls. Leaf area and colour of both types of transgenic plants were also similar to controls. In comparison with non-transformed controls, the expression of the MT-L2 gene increased two- to four-fold in light-induced SSU-*ipt* plants (Fig. 4). Similar results were obtained in the HS-*ipt* plants following heat shock *ipt*-induced expression (data not shown). Surprisingly, 4 m*M* $CuSO_4$ stress for 7 days did not appreciably effect leaf MT-L2 mRNA levels in the SSU-*ipt* plants (Fig. 4). Leaf 9 and 10 contained similar levels of MT-L2 mRNA in both stressed and unstressed conditions. Similar results were obtained in cell culture experiments with *N. plumbaginifolia* HS-*ipt* cells (data not shown).

Following $CuSO_4$ stress for 7 days (4 m*M* $CuSO_4$), GUS control and SSU-*ipt* *N. tabacum* plants were recovered, their tissues pooled into developmental categories: stem; lowest 2–3 leaves (very old); the next higher three leaves (old); the next higher three leaves

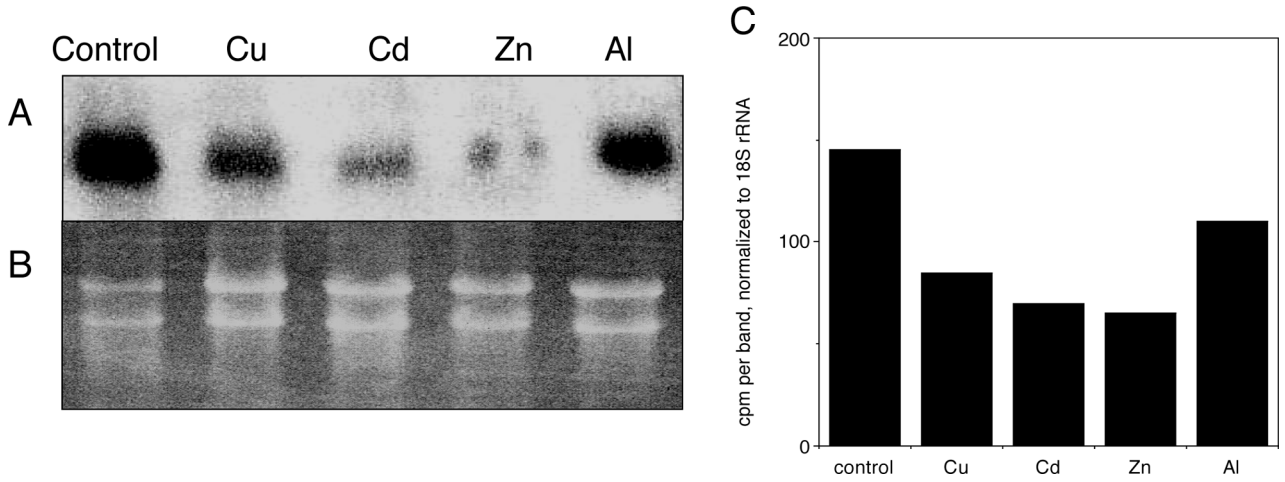

Fig. 2. Northern blot analysis of the MT-L2 transcript in normal *N. plumbaginifolia* leaf strips untreated or treated for 30 h with 50 μ*M* $CuSO_4$ (Cu), 100 μ*M* $CdCl_2$ (Cd), 1000 μ*M* $ZnCl_2$ (Zn) or 100 μ*M* $AlCl_2$ (Al). A, Hybridization to the MT-L2 probe. B, Gel photograph of ethidium bromide-stained total RNA to verify equal RNA loading per lane. C, Counts per MT-L2 band normalized relative to 18S rDNA hybridization to the 18S RNA.

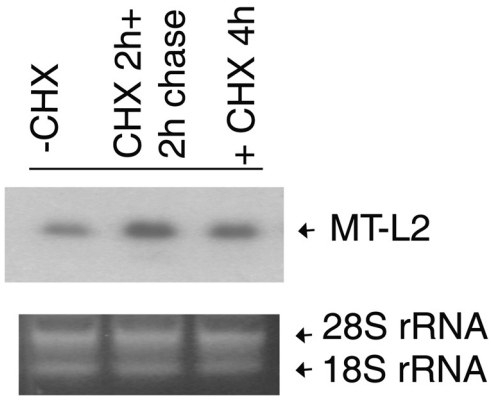

Fig. 3. Non-transformed *N. tabacum* cv. SR1 leaf accumulation of MT-L2 transcripts following treatment with cycloheximide (CHX). Leaf segments were floated on Murashige and Skoog medium +/– cycloheximide (1 μg ml^{-1}) (CHX) for 4 h. Some leaves were washed (chased) after 2 h and allowed to recover for 2 h. Fifteen micrograms of total RNA was loaded per lane (as seen in the ethidium bromide stained gel showing the 28S and 18S rRNA. This experiment was repeated.

(middle); and the three or four highest leaves (young). Tissue was ashed and analysed for the accumulation of copper. Expression of the *ipt* gene and subsequent enhanced cytokinin production led to an eight-fold increase in copper accumulation in the most mature (very old) leaves in comparison with the non-transformed controls (Fig. 5).

Lipid peroxidation

To investigate the contribution of oxidative stress during copper exposure in tobacco, samples of leaves stressed with copper were analysed for lipid peroxidation. Transgenic SSU-ipt plants contained less lipid peroxidation than controls. Furthermore, copper stress stimulated 56% more lipid peroxidation than unstressed controls (Fig. 6).

Discussion

MT-L2 gene a member of a small gene family in tobacco

In higher plants such as Arabidopsis, metallothioneins are generally found as members of a gene family (García-Hernández et al. 1998, Cobbett and Goldsbrough 2002). Within that family, the four Arabidopsis MTs classes can be characterized as being expressed in the root (MT1), shoot (MT2), leaves and fruit (MT3) and throughout the plant (MT4) (Cobbett and Goldsbrough 2002). Recently MT promoter fusions to reporter genes suggested that MT1a and MT2b are localized in phloem of both roots and leaves, whereas MT2a and MT3 are highly copper inducible and mostly expressed in root tips and young leaves (Guo et al. 2003). It would be expected that MT1, MT3 and MT4 homologs are also present in Nicotiana. Three rice MT-L2 isoforms are differentially expressed in differing tissues, suggesting expression may be balanced in many tissues of some higher plants (Wong et al. 2004).

MT-L proteins in higher plants

Most higher plant MT-L2 proteins and the CUP-1 yeast metallothionein contain N- *and* C-terminal metal-binding domains separated by a short linker region. The protein structure must also account for the interaction of the MT

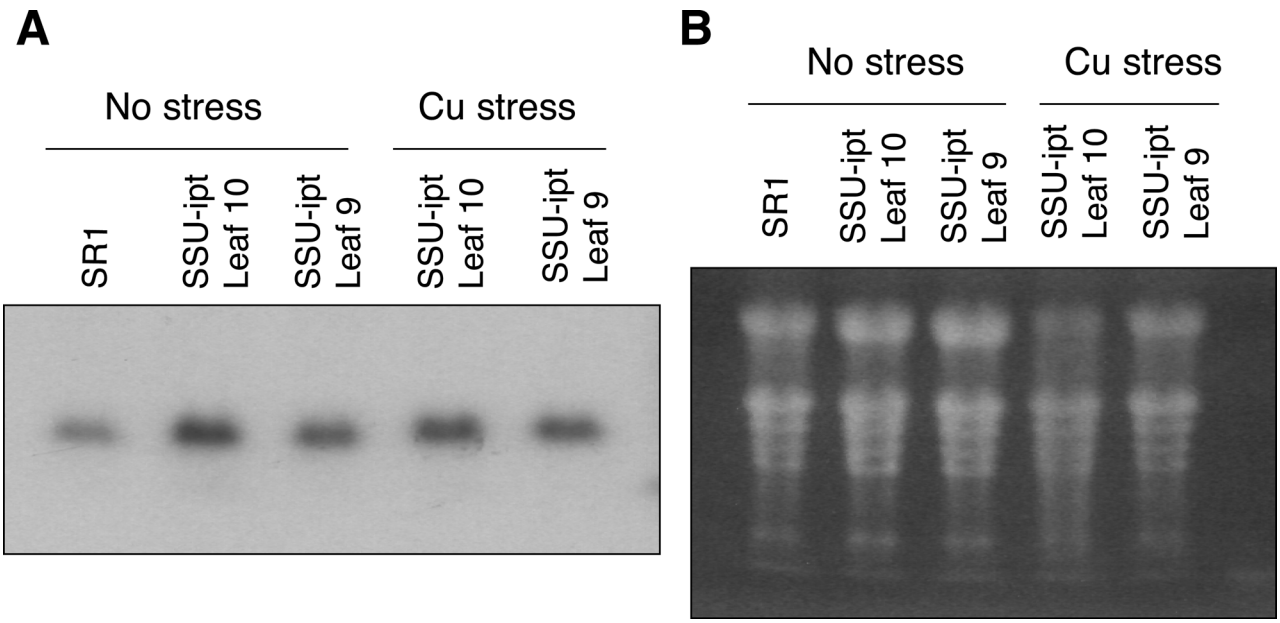

Fig. 4. A, MT-L2 gene expression in non-transformed (lane 1) and SSU-*ipt N. tabacum* plants (lanes 2–5). In lanes 1–3 plants were not stressed whereas RNA in lanes 4 and 5 was from copper stressed plant leaves. B, Image of the ethidium bromide stained total RNA to verify total RNA loading. This experiment was repeated.

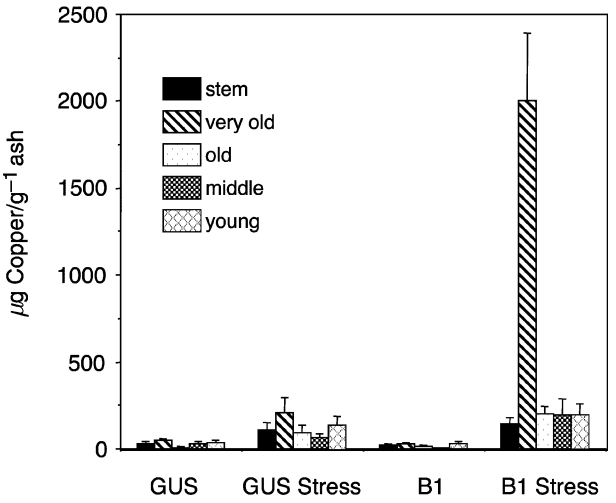

Fig. 5. Leaf copper levels in *N. tabacum* SR1 GUS transformed and SSU-*ipt* (B1) plants. Data is presented as micrograms copper per gram ash weight and represents the mean and standard error of duplicate treatments of plants grown in three separate experiments.

with glutathione, a potent biological reductant that allows copper binding (Brouwer et al. 1993). Within the plant MT-L genes, differing arrangements of cysteines form the basis of MT-L gene classification, perhaps reflecting subtle differences in protein function (Cobbett and Goldsbrough 2002). The role of the metallothionein CUP-1 in yeast is to confer copper metal tolerance (Hamer et al. 1985, Jensen et al. 1996). To test whether higher plant MT-L2 genes are involved in metal tolerance, several groups have over-expressed MT genes in selfed and genetically stable generations of Arabidopsis and tobacco. The results demonstrated that transgenic plant tolerance to metal was not enhanced above non-transformed controls, however, metal accumulation was often elevated (Evans et al. 1992, Thomas et al. 2003).

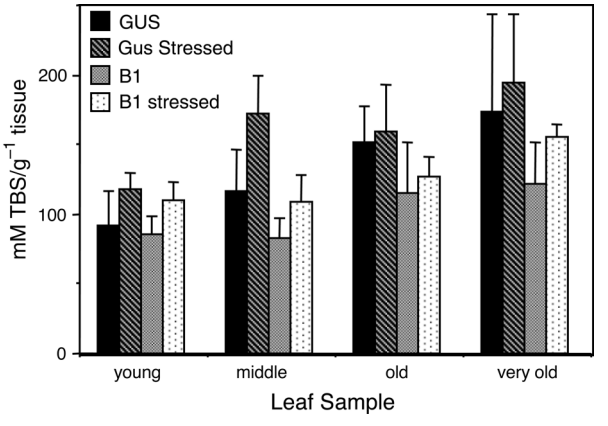

Fig. 6. Leaf lipid peroxidation in unstressed and copper stressed *N. tabacum* SR1 GUS transformed and SSU-*ipt* (B1) plants. The plant tissues analysed were the same as shown in Fig. 5. Each data point represents the average MDA level of pooled plants from three separate experiments. Standard error is shown with bars.

Conversely, cosuppression of native MT-L genes may help explain reports where enhanced metal tolerance and no effect on metal uptake was observed in transgenic plants over-expressing a mammalian MT (Elmayan and Tepfer 1994). The results presented here argue that MT-L2 mRNA levels diminish under copper stress in tobacco, suggesting these small proteins do not function as a tolerance factor in all higher plants.

Nutrition is a more compelling possible function for higher plant MT-L2s, somewhat akin to the yeast CRS5 metallothionein-like gene (Jensen et al. 1996). The tobacco MT-L2 studied here appeared relatively stable. MT-L2 mRNA levels increased significantly after 4 h of CHX treatment, about twice the mRNA level in the 2 h CHX and 2 h chase treatment (Fig. 3). The MT-L2 transcript half-life of about 2 h is comparable to either GUS or yeast metallothionein transcripts (DeRocher et al. 1998, Thomas et al. 2003), perhaps expected for a metabolically important protein. The MT-L2 mRNA was also localized throughout the plant, at similar levels in leaves and at slightly higher levels in the stems (Fig. 1 and Thomas et al. 2003). Generally constitutive in nature, the MT-L2 mRNA expression patterns combined with the loss of transcript upon metal stress (Figs 1 and 4) are consistent with the notion that MT-L2 genes are involved in copper use for nutrition and metabolism (Cobbett and Goldsbrough 2002). Recent study in mammalian systems suggests that MTs may also play a modulator role in ATP synthesis and obesity (Ye et al. 2001).

Normal MT-mRNA expression

Our findings suggest tobacco normally decreases the MT-L2 mRNA levels following a copper stress. Similarly, a second tobacco MT-L2 family member increased in mRNA accumulation following wounding and viral stress, but only slightly by copper stress (Choi et al. 1996). These authors asserted that phytotoxic levels of copper resulted in a decline in expression to pre-stressed levels (Choi et al. 1996). Using a similar stress strategy used in this report (soil grown mature plants and 4 m*M* $CuSO_4$ for 1 week), it is noteworthy that transgenic tobacco expressing the yeast CUP-1 also showed a decline in CUP-1 transcripts after copper stress (Thomas et al. 2003). It is unknown whether tobacco represses MT-L2 transcription levels or promotes mRNA degradation, processes consistent with a phytotoxic copper response. However the question remains, why some plant species demonstrate enhanced mRNA accumulation of MT-L2 genes whereas others show mRNA levels that decline under copper stress (Rauser 1999).

Oxidation and MTs

Coping with copper requires that the plant discern the redox-active metal as an important nutrient or, depending on dose, an environmental stress. Copper may become toxic to plants in several ways. This redox-active metal can produce reactive oxygen species by way of the

Harber–Weiss and Fenton reactions (Sandermann and Böger 1980, Elstner et al. 1988). Copper may displace other metals in metalloproteins (like zinc fingers), thus altering the function of a transcription factor. Finally excessive uptake of any salt (including copper sulphate) can change the cellular osmotic potential.

Modulated in part by MT levels, oxidation itself may represent an important developmental signal (Storz et al. 1990). Oxidation and the generation of ROS are consequences of normal metabolism (Foyer and Noctor 2003). Copper stress can further exacerbate normal oxidation and free radical formation, as evidenced by enhanced lipid peroxidation compared to unstressed controls (Fig. 6) (Sandermann and Böger 1980). Used for protection, MTs themselves are considered antioxidants in mammalian systems (Lazo et al. 1998). In rice, OsMT2b-overexpressing plants showed increased susceptibility to both bacterial blight and blast infection with concurrent decline in H_2O_2 production (Wong et al. 2004). Furthermore, purified OsMT2b protein demonstrated greater protection from hydroxyl radical-mediated salicylate hydroxylation compared to the vector (GST) alone (Wong et al. 2004). Hamer has proposed the regulation of intracellular redox state and detoxification of ROS may be the principal activity of these copper-binding metallothioneins (Hamer et al. 1985).

It follows that to understand the physiological impact of copper stress on whole plants within the context of MT-L2 gene expression, the developmental status and tissue type being analysed of the plant must be considered (Kawashima et al. 1992, Thomas et al. 2004). While copper hyperaccumulator plant species are not prevalent, tolerant plants such as *Silene vulgaris* and *Mesembryanthemum crystallinum* have probably evolved enhanced antioxidant strategies against salt and osmotic stress, some that may also provide protection against copper stress (Thomas et al. 1998, Van Hoof et al. 2001).

Cytokinin, development and copper stress

A great deal of data suggests that the plant growth regulator cytokinin may protect against senescence in young vegetative tissues (Taiz and Zeiger 2002). Depending on the dose, cytokinins are also implicated in the modulation of several stress responses (Thomas and Bohnert 1993, Harding and Smigocki 1994, Thomas et al. 1995a, b). This apparent contradiction may be due to the dose-dependant and tissue-specific responses characteristic of cytokinin treatment (Werner et al. 2001). Further complicating the understanding of biologically relevant mechanism(s), cytokinins can have opposing effects on meristematic regions depending on their tissue type (root versus shoot) (Werner et al. 2003). For example, leaf CAM-specific phosphoenolpyruvate carboxylase (PPC1) gene expression was inhibited by cytokinin application to aerial portions but enhanced by cytokinin application to roots (Thomas and Bohnert 1993, Peters et al. 1997). What is clear is that cytokinin can profoundly affect plant development (Taiz and Zeiger 2002).

During vegetative senescence and flowering, cytokinin is responsible for playing a role (s) in nutrient partitioning (Lejeune et al. 1988). Cytokinin biosynthesis in leaves and subsequent transport in the phloem was observed in plants approaching flowering (Taylor et al. 1990). Cytokinin levels also increased in shoot meristems and they are correlated with floral transition (Corbesier et al. 2003). Support for a role of cytokinins in flowering comes from genetically engineered Arabidopsis plants with enhanced cytokinin breakdown activities. Lack of cytokinin resulted in plants with a 3-month delay in flowering (Werner et al. 2001). Even post-reproduction, cytokinins help maintain a reducing environment and viability in developing and germinating seeds (Gidrol et al. 1994).

We speculate that during a copper challenge, copper accumulation in older leaves of cytokinin-overproducing plants preserves the younger growing vegetative tissues, advantageous for survival of the plant. Protection from oxidation may also be the result of developmental modifications facilitated by long-term exposure to cytokinins. Constitutive production of cytokinin (as in SSU-*ipt* plants) throughout development (7–8 weeks) has been shown to alter source–sink relationships (Guivarc'h et al. 2002). *Ipt*-expressing plants either copper stressed or unstressed contained less peroxidation than the respective controls (Fig. 6). Therefore, by producing excess cytokinin and enhancing MT-L2 mRNA concentration (and presumably MT-L2 protein) in a less oxygenic environment, metal exchange or accumulation in older tissues during a copper stress could result (Fig. 5). MT-L2 mRNA levels were elevated 4 and 22 h post-heat shock in non-transformed and HS-*ipt* *N. plumbaginifolia* leaf segments (data not shown). However, copper levels were not enhanced above controls in these heat-shocked samples (data not shown). Older leaves showed increased copper sequestration in comparison with controls in CUP-1 over-expression in tobacco (Thomas et al. 2003), suggesting that global resource (copper) partitioning into older tissues may require MT-L2 protein accumulation, or at least enhanced antioxidant activity over extended developmental time.

Besides regulatory effects on the redox status of higher plants, cytokinin and copper may represent opposing activities upon the cell cycle. Cytokinin positively regulates mitosis in higher plants (Taiz and Zeiger 2002), likely via a Cdc2 kinase and stimulation of cyclin D (Riou-Khamlichi et al. 1999). Conversely, H_2O_2 or stress inducers copper or NaCl negatively regulate the cell cycle via phosphorylation events (Taybi and Cushman 1999, Kovtun et al. 2000). Recently, one 42-kDa MAPK-like kinase activity increased in response to copper was returned to pre-stress levels with the addition of an antioxidant (Yeh et al. 2003). Thus, cytokinin and copper stress may both be acting on signalling, via the phosphorylation status of CDKs and cyclins of the cell cycle.

Our current hypothesis suggests that in tobacco, the copper-mediated MT-L2 mRNA decline in response to copper stress is a consequence of copper-induced decrease in cytokinin. This view suggests a different mechanism of MT mRNA regulation in higher plants when compared to yeast (Winge et al. 1998). Upon exposure to excessive water or salt stresses, cytokinin levels also decline (Thomas and Bohnert 1993) and cytokinin oxidase activity increases, ultimately leading to cytokinin breakdown (Manju et al. 2001). Thus, MT-L2 mRNA accumulation in *ipt*-expressing transgenic plants could be a consequence of cytokinin-stimulated MT-L2 mRNA transcription and/or stability. This idea is consistent by cytokinin protection from lipid peroxidation (Fig. 6), itself an outcome of ROS.

Cytokinins have a central role as a master regulator in development, possibly inducing MT-L2 gene expression and/or contributing to a more reduced internal plant environment. Variation in cytokinin titres may explain species-specific variations in MT-L2 mRNA responses to metal stress (Kawashima et al. 1992, Zhou and Goldsbrough 1994, Choi et al. 1996, Rauser 1999). Should plants preserve cytokinin levels via altered copper transport or sequestration mechanisms or enhanced cytokinin production, MT-L2 expression in response to copper stress could be maintained or perhaps even increased. Tissue-specific differences in cytokinin amounts could also account for some differential MT-L mRNA expression (García-Hernández et al. 1998). Understanding the copper partitioning in older leaves of cytokinin-expressing and copper-stressed plants may be more complicated. Cytokinin could either affect MT-L2 mRNA and/or metal transporter proteins, thereby changing source–sink relations for the micronutrient metal. These changes may be evident throughout development. Detailed cytokinin determinations following copper addition would help verify this hypothesis. In addition, examination of MT-L2 mRNA profiles in Arabidopsis mutants with deficient cytokinin synthesis would further address whether MT levels are dependent on threshold cytokinin levels.

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References

Brouwer M, Hoexum-Brouwer T, Cashon R (1993) A putative glutathione-binding site in CdZn-metallothionein identified by equilibrium binding and molecular modeling studies. Biochem J 294: 219–225

Choi D, Kim H, Yun H, Park J, Kim W, Bok S (1996) Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* and its induction by wounding and tobacco mosaic-virus infection. Plant Physiol 112: 353–359

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metals detoxification and homeostasis. Annu Rev Plant Biol 53: 159–183

Corbesier L, Prinsen E, Jacqmard A, Lejeune P, Onckelen H, Périlleux C, Bernier G (2003) Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. J Exp Bot 54: 2511–2517

DePaula M, Pérez-Otaola M, Darder M, Torres M, Frutos G, Martínes-Honduvilla C (1996) Function of the ascorbate-glutathione cycle in aged sunflower seeds. Physiol Plant 96: 543–550

DeRocher EJ, Vargo-Gogola T, Diehn S, Green P (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol 117: 1445–1461

Elmayan T, Tepfer M (1994) Synthesis of a bifunctional metallothionein/β-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium. Plant J 6: 433–440

Elstner E, Wagner G, Schutz W (1988) Activated oxygen in green plants in relation to stress situations. Curr Top Plant Biochem Physiol 7: 159–187

Evans K, Gatehouse J, Lindsay W, Shi J, Tommey A, Robertson N (1992) Expression of the pea metallothionein-like gene PsMtA in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: implications for PsMtA function. Plant Mol Biol 20: 1019–1028

Foyer C, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119: 355–364

Freeman J, Peisach J (1989) Resistance of cultured hepatoma cells to copper toxicity: Purification and characterization of the hepatoma metallothionein. Biochem Biophys Acta 992: 145–154

García-Hernández M, Murphy A, Taiz L (1998) Metallothioneins 1 and 2 have distinct but overlapping expression patterns in Arabidopsis. Plant Physiol 118: 387–397

Gidrol X, Lin W, Degousee N, Yip S, Kush A (1994) Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J Biochem 224: 21–28

Gilbert D (1990–96) SeqPup. In: Ed, Version 0.6. Biology Department, Indiana University, Bloomington, IN

Guivarc'h A, Rembur J, Goetz M, Roitsch T, Moin M, Schmülling T, Chriqui D (2002) Local expression of the ipt gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) axillary buds establishes a role for cytokinins in tuberization and sink formation. J Exp Bot 53: 621–629

Guo W-J, Bundithya W, Goldsbrough P (2003) Characterization of the Arabidopsis metallothionein gene family: tissue-specific expression and induction during senescence and in response to copper. New Phytol 159: 369–381

Hamer D, Thiele D, Lemont J (1985) Function and autoregulation of yeast copperthionein. Science 228: 685–690

Harding S, Smigocki A (1994) Cytokinins modulate stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. Physiol Plant 90: 327–333

Himelblau E, Amasino R (2001) Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. J Plant Physiol 158: 1317–1323

Jensen L, Howards W, Strain J, Winge D, Culotta V (1996) Enhanced effectiveness of copper ion buffering by CUP1 metallothionein compared with CRS5 metallothionein in *Saccharomyces cerevisiae*. J Biol Chem 271: 18514–18519

Kawashima I, Kennedy T, Chino M, Lane B (1992) Wheat Ec metallothionein genes: Like mammalian Zn^{2+} metallothionein genes are conspicuously expressed during embryogenesis. Eur J Biochem 209: 971–976

Klassen C, Liu J, Choudhuri S (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol 39: 267–294

Kovtun Y, Chiu W-L, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Nat Acad Sci USA 97: 2940–2945

Lazo J, Kuo S-M, Woo E, Pitt B (1998) The protein thiol metallothionein as an antioxidant and protectant against antineoplastic drugs. Chem Biol Interact 111–112: 255–262

Lejeune P, Kinet J-M, Bernier G (1988) Cytokinin fluxes during floral induction in the long day plant *Sinapis alba* L. Plant Physiol 86: 1095–1098

Manju R, Kulkarni M, Prasad T, Sundarshana L, Sashidhar V (2001) Cytokinin oxidase activity and cytokinin content in roots of sunflower under water stress. Indian J Exp Bot 39: 786–792

Murphy A, Zhou J, Goldsbrough P, Taiz L (1997) Purification and immunological identification of metallothioneins 1 and 2 from *Arabidopsis thaliana*. Plant Physiol 113: 1293–1301

Page T, Griffiths G, Buchanan-Wollaston V (2001) Molecular and biochemical characterization of postharvest senescence in brocoli. Plant Physiol 125: 718–727

Peters W, Beck E, Piepenbrock M, Lenz B, Schmitt J (1997) Cytokinin as a negative effector of phosphoenolpyruvate carboxylate induction in *Mesembryanthemum crystallinum*. J Plant Physiol 151: 362–367

Rauser W (1999) Structure and function of metal chelators produced by plants, the case for organic acids, amino acids, phytin and metallothioneins. Cell Biochem Biophys 31: 19–48

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray J (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science 283: 1541–1544

Sandermann G, Böger P (1980) Copper-mediated lipid peroxidation processes in photosynthetic membranes. Plant Physiol 66: 797–800

Smart C, Scofield S, Bevan M, Dyer T (1991) Delayed leaf senescence in tobacco plants transformed with tmr, a gene responsible for cytokinin production in *Agrobacterium*. Plant Cell 3: 647–656

Smigocki A (1991) Cytokinin content and tissue distribution in plants transformed by a recombinant isopentenyl transferase gene. Plant Mol Biol 16: 107–115

Storz G, Taraglia L, Ames B (1990) Transcriptional regulation of oxidative stress-inducible genes: Direct activation by oxidation. Science 248: 189–194

Taiz L, Zeiger E (2002) Plant Physiology, 3rd edn. Sinauer Associates Inc, Sunderland, MA

Taybi T, Cushman J (1999) Signaling events leading to Crassulacean acid metabolism induction in the common ice plant. Plant Physiol 121: 1–11

Taylor J, Thompson B, Pate J, Atkins C, Pharis R (1990) Cytokinins in the phloem of white lupin (*Lupinus albus* L.). Plant Physiol 94: 1714–1720

Thiele D (1992) Metal-regulated transcription in eukaryotes. Nucl Acid Res 20: 1183–1191

Thomas J, Bohnert H (1993) Salt stress perception and plant growth regulators in the halophyte, *Mesembryanthemum crystallinum*. Plant Physiol 103: 1299–1304

Thomas J, Davies E, Malick F, Endreszl C, Williams C, Abbas M, Petrella S, Swisher K, Perron M, Edwards R, Osenkowski P, Urbanczyk N, Wiesend W, Murray K (2003) Yeast metallothionein in transgenic tobacco promotes copper uptake from contaminated soils. Biotechnol Prog 19: 273–280

Thomas J, Malick F, Endreszl C, Davies E, Murray K (1998) Distinct responses to copper stress in the halophyte, *Mesembryanthemum crystallinum*. Physiol Plant 102: 360–368

Thomas J, Perron M, Davies E (2004) Genetic responsiveness to copper in the Ice Plant, *Mesembryanthemum crystallinum*. Plant Sci 167: 259–266

Thomas J, Smigocki A, Bohnert H (1995a) Light induced expression of ipt from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. Plant Mol Biol 27: 225–235

Thomas J, Smigocki A, Bohnert H (1995b) Erratum. Plant Mol Biol 28: 965

Van Hoof N, Hassinen V, Hakvoort H, Ballintijn K, Schat H, Verkleij J, Ernst W, Karenlampi S, Tervahauta A (2001) Enhanced copper tolerance in *Silene vulgaris* (Moench) garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Plant Physiol 126: 1519

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15: 2532–2550

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Nat Acad Sci USA 98: 10487–10492

Winge D, Jensen L, Srinivasan C (1998) Metal-ion regulation of gene expression in yeast. Curr Opin Chem Biol 2: 216–221

Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135: 1447–1456

Ye B, Maret W, Vallee B (2001) Zinc metallothionein imported into liver mitochondria modulates respiration. Proc Nat Acad Sci USA 98: 2317–2322

Yeh C-M, Hung W-C, Huang H-J (2003) Copper treatment activates mitogen-activated protein kinase signalling. Plant Physiol 112: 353–359

Zhou J, Goldsbrough P (1994) Functional homologues of fungal metallothionein genes from Arabidopsis. Plant Cell 6: 875–884

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